Development of RNAi based nanoformulation against *Mungbean Yellow Mosaic Virus* in *Vigna mungo* (L.)

Synopsis

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INTRODUCTION

The common name of *Vigna mungo* (L.) is black gram; it is a leguminous crop with self-pollinated short life cycle. Its genome is composed of 11 pair of chromosomes (2n=2x=22). On the bases of previous researches, it can be said that before 2.7 million years ago *Vigna mungo* and *Vigna radiata* have been diverged (Arumunganathan and Earle, 1991; Pootakham *et al.,* 2021). Black gram is rich in nutritious supplements such as protein, starch, iron, vitamins and minerals (Akinyele *et al.,* 1989). It is mainly found in tropical and subtropical Indian region and due to its yield quantity, it is the third major crop of Indian subcontinent. The continuous increase in black gram diseases is becoming a matter of concern in south Asian regions. Several pathogens infect plants but majority of crop loss occurs due to the viral infection (Marwal *et al.,* 2020). They are also responsible for the economic loss because of the lack of an effective approach to cop up with viral infection (Singh *et al.,* 2014; Cai *et al.,* 2019).

Virus infection alters the gene expression of host plant and also affects the metabolic activity of the target organism. The symptoms in host plant start to appear after virus invading, the host component led to the change in molecular pattern of host plant. Due to the viral infection, mRNA of host plant gene downregulates and results in the systemic infection. The downregulation of mRNA is a long-term procedure because it occurs for several weeks and responsible for the severe mRNA loss for housekeeping genes (Havelda *et al.,* 2008). Viruses generally cause acute and chronic symptoms but some viruses do not cause any infection due to their persistent lifestyle. Therefore, they have not been studied much (Roossinck, 2010). The common symptoms of virus infection are mosaic, mottling, underdeveloped fruit, and wrinkled leaves. Virus also halts the physiological activity of host plant which led to the symptoms like necrosis, reduction in plant growth and can also cause plant death (Yang *et al.,* 2018).

Viruses belongs to Begomoviruses family majorly affect *Vigna mungo.* viruses such as *Mungbean yellow mosaic Indian virus* (MYMIV), *Mungbean yellow mosaic virus* (MYMV) and *Dolichos yellow mosaic virus* (DoYMV) are known for major viral infection in black gram (Quazi *et al.,* 2007). *Mungbean yellow mosaic virus* not only infects the black gram even it also infects other leguminous crops such as soybean, cowpea, etc. (Dhingra and Chenulu 1985; Qazi *et al.,* 2007). The MYMV is the member of Geminiviridae with particle size 20×30nm. The genome of MYMV contains single standard

spherical DNA which is encapsulated with protein coat, its genome size is about 2.8 Kb. The white flies are the vector for the transmission of virus. Both male and female white flies known for the viral transmission in black gram but female white flies are responsible for more effective transmission (Coata 1976).

RNA interference (RNAi) is an amazing technique that offers effective disease management strategy (Ballarin-Gonzalez and Howard 2012). It is actually a post-Transcriptional gene Silencing (PTGS) technique. In this technique conserved sequences are generated to silence the target gene due to this feature of RNAi is considered as the sequence specific action towards double stranded RNA. It offers the resistance against pathogenic genome (Hannon 2002). RNAi is a two steps technique; the previous step is involved in the Dicer mediated breakdown of dsRNA and this breakdown step led to the formation of small RNAs known as siRNA. In the second step RISC (RNA-induces silencing complex) is formed in which siRNA is combined with RNase complex. This RISC factor is responsible for the target mRNA breakdown. Rather than Dicer other factors such as dsRNA endonucleases, helicase, RNA-dependent RNA polymerase are also involved (Agrawal *et al.*,2003; Wilson & Doudna, 2013). In 1990, Napoli and colleagues provided the first report of post-transcriptional gene silencing (Napoli *et al.*, 1990). To improve nutritional quality and increase pest resistance, plant transformation is an effective approach, but this technique also involves major challenges in gene delivery.

In plants RNAi is an autoimmune response against foreign nucleotide. To suppress the RNAi mediated plant immune response, virus encodes "silencing suppressors" (VSRs) such as 2b protein encoded by cucumber mosaic virus (CMV), P19 protein of *Carnation Italian ringspot virus* (CIRV) and helper component-proteinase (HC-Pro) of some potyvirus are known to obstruct the RNAi mediated autoimmune response of plant (Guo *et al.*, 2002; Lakotos *et al.*, 2006; Ding and Voinnet, 2007; Konakalla *et al.*, 2016).

Nanotechnology is an emerging approach to overcome these challenges and provide appropriate medium for delivery of biomolecule due to the unique ability and physiochemical property of nanoparticles (Kumar *et al.*, 2020). Nanoparticles are emerging as efficient tool specifically in the biomedical field. Viral nanoparticle also known as VNPs which are derived from various kinds of viruses, due to this feature they are known as natural bio-nanomaterials. Plant VNPs provide more

efficient comparatively and overcome more challenges instead of other VNPs (Steinmetz, 2010). Genetic engineering is an effective approach to enhance the productivity of plant and manipulate the trait of interest in target sessile organism. The nanotechnology offers the comparatively high and diversely effective biomolecule delivery because of their unique physiochemical property and well penetration of thick cell wall. Nanocarriers are also considered as the effective agents against pathogenic microorganism such as pest or bacteria (Kumar *et al.,* 2020). Biodegradable nanoparticles mediated delivery emerges as a versatile tool for the delivery of low molecular weight compound, DNA, Plasmid, etc. (Hans and Lowman 2002; Panyam and Labhasetwar, 2003).

RNAi provide target selection; it is an efficient tool for pest control. Nowadays, nanocarriers are used to deliver RNAi (Wu *et al.,* 2014; Elhaj Baddar *et al.,* 2020). Nanocarriers are known for their unique property such as enhanced stability, cost effective mass production of dsRNA and it is also considered as an economic way of dsRNA delivery (Mitter *et al.,* 2017).

In past century, several researches for drug delivery aimed to increase efficacy of particular drugs nanoparticles and reduce the side effect (Aprahamian *et al.,* 1987). Biodegradable nanoparticles are considered as an effective carrier for site specific transfer of gene and other biomolecules as well as drugs and vaccine in *vivo* due to their convenient release profiles. Numerous biodegradable nanoparticles have been reported some of them are PLGA (Poly-D-L-lactide-co-glycolide), polyleactic acid (PLA), poly-E-caprolactone (PCL), chitosan, gelatine, poly-alkyl-cyano-acrylates (PAC) etc. (Mahapatro *et al.,* 2011). Chitosan is a natural polymer and also approved by the Generally recognised as safe (GRAS) United States Food and Drug Administration. Numbers of methods are also available to prepare chitosan nanoparticles such as reverse micellar method, ionic cross-linking method, precipitation, covalent cross linking and emulsion-droplet coalescence method (Garg *et al.,* 2019).

For the delivery of biomolecule, delivery vehicle should have several necessary features such as high efficiency for continuous activation of RNAi, targeted delivery; maintain stable structure and it should be non or less toxic. There are number of nanoparticles that have been reported as biomolecule delivery vehicle. Among which PLGA are approved by Food and Drug Administration in 1969 which make this nanoparticle a safe drug targeting nanoparticle. PLGA nanoparticles share specific feature as they are solid in nature and are also able to protect nucleic acid from degradation which make them

effective and efficient delivery vehicle for biomolecule like RNAi. (Jones *et al.*,1997; Luo *et al.*,1999; Shea *et al.*,1999; Pirollo *et al.*,2007; Chen *et al.*,2010). The RNAi delivery via transgenic approach is in market but the spray Induce Gene Silencing could be a fascinating approach which work as alternative to transgenic technique and also cost effective. Plants indirectly uptake the dsRNA through plant cells (Cagliari *et al.*, 2019). MYMV infection in *Vigna mungo* is responsible for 50-60% loss in crop yield as well as quality loss in Dayalbagh field of Agra region which increases the concern. Continuous decrement in the crop quality and quantity requires attention to deal with viral infection. dsRNA loaded nanocarriers could be an effective approach to reduce crop loss due to virus infection.

Relevance and outcome of the study: This work provides the new insights in the field of agriculture disease management via using a biodegradable nanocarrier as a delivery vehicle for dsRNA. Our aim is to achieve decrement in the use of chemical and harmful substances in agro-ecological system.

OBJECTIVES

Through our research we make an attempt to develop low-cost eco-friendly technique to minimise the crop loss due to viral disease which led to the crop loss. We want to develop an efficient technique which can be used by farmers at large scale to overcome the economic loss.

To full fill the aims of our research the main objectives will be:

- Identification of *Mungbean Yellow Mosaic virus* (MYMV) *in Vigna mungo* from Dayalbagh field Agra.
- Construction of precursor siRNA against target gene.
- Development of Chitosan & PLGA nanoparticles and conjugation of precursor siRNA to nanoparticles.
- Efficacy analysis of nanoformulation against MYMV.

LITERATURE REVIEW

Nowadays to cop up with pathogen caused disease, two techniques are renowned and provide effective results, double-stranded (dsRNA) nanoclaysheets and CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats) (Ahsan and Yuanhua, 2021). Almost all eukaryotes show RNAi mediated defence mechanism as their natural defence machinery against foreign molecule. The process

of RNAi can activate artificially through viruses and transgenes. For more than a decade application of exogenous RNA is a tool for the induction of RNAi in Plants (Uslu and Wassenegger, 2020). Outer protein coat of plant virus that is known as capsid can be used as bio templates for the synthesis of nanomaterials so for further use in the field of bioscience (Alemzadeh *et al.*,2018). nowadays, nanotechnology touches the heights of popularity in agriculture. Nanotechnology based plant viral disease detection kits are also available in market which allows the early detection of plant viral disease (Perez-de-Luque and Rubiales 2009).

On the basis of research conducted by Cai and colleague (2019), revealed that some nanoparticles possess antiviral property such as zinc oxide (ZnONPs) and silica based (SiO2NPs). The pretreatment of TMV particles with zinc oxide or silica nanoparticles led to the particle aggregation in 2 hours. When the prepared mixture was inoculated onto the infected leaves for two days led to the reduction of the viral infection. But the continuous use not really able to halt the viral replication even though the systemic resistance of plant system also got generated due to the continues application of nanoparticles. The systemic plant resistance led to the generation of the ROS, peroxidase and catalase activity. Cai and colleague also conclude that due to the ZnONPs nanoparticles some of the phytohormones also get increase.

Demirer and colleagues (2020), prove successful delivery of siRNA into plant cell of *N. benthamiana* to target the green fluorescent protein transgene in transgenic *N. benthamiana* via using a nanocarrier, single-walled carbon nanotube (SWNTs) and they were successful in silencing the target gene.

Das and colleagues (2015), target the NP coated with RNAi to control over *Aedes aegypti* larvae by silencing SNF7 and SRC. The feeding food contains dsRNA coated on nanoparticles the decrease in the mRNA level have been reported in chitosan coated with dsAaSNF7 and dsAaSRC feeding larvae in nanoparticle-dsRNA treated larvae they quantify the efficiency of knockdown via qRT-PCR. They tested three nanoparticles for knockdown of gene of interest chitosan, carbon quantum dot (CQD) and silica among them CQD prove more effective delivery vehicle for dsRNA.

Tenllado and Diaz-Ruiz (2001), work suggested that dsRNA designed from viral sequences play sufficient role against virus infection. They were successfully proved that the dsRNA intermediate

perform role as post transcriptional gene silencing initiator so the viral RNA targets for the degradation phenomenon. They confirmed the gene silencing through dsRNA to obtain resistance against three groups of virus tobamovirus, potyvirus, and alfamovirus. The reduction in the formation of virus mediated lesion had seen after the co-inoculation of dsRNA homologous to target gene.

Bonfim and colleagues (2017), constructed RNAi for silencing the viral gene (AC1) which is particularly associated with whiteflies transmitted virus in *Phaseolus vulgaris* L. out of 18 transgenic lines prepared one line shows significant resistance about 93% of them didn't shows viral symptoms.

Ibrahim and colleagues (2017), successfully constructed the RNAi-based plasmid which contains engineered interfering cassette, aid in the dsRNA generation which allow the successful targeting of *v-ATPase* transcript of whitefly. Their work suggests effective results.

The half-life of naked dsRNA in serum is an hour (Bartlett and Davis, 2007) to cop up with limitation Mitter and colleagues (2017), use nanosheets to transfer dsRNA. They demonstrate that the dsRNA used to load on designed nanosheets, layered double hydroxide clay nanosheets (LDH) which do not allow the washing off dsRNA as well as easily detectable on spray. The ds RNA loaded nanosheets termed as BioClay. These BioClay are affordable and provide significant result against virus for almost 20 days and also environmentally safe. When BioClay spread on plants, the environmental water vapours and CO_2 are combinedly responsible for the steady release of dsRNA.

Exogenous application of dsRNA by Konakalla and colleagues (2016), provides favourable result. They prove that the *Tobacco mosaic virus* (TMV) infection can be affected by RNAi despite it is synthesized in vitro or apply externally on plant. The exogenous dsRNA successfully processed in plant system via forming of Dicer-like-protein complex. They test the dsRNA molecule result on 20 tobacco plants in four sets of following treatments: TMV with dsRNA_p126, TMV with dsRNA_CP, TMV as negative control and water as positive control. Both treatments were successful in TMV infection resistance. Their data suggest that those plants treated with dsRNA_p126 show higher resistance than those treated with dsRNA-CP. Both treated plants show no symptoms of delayed symptoms.

For the delivery of siRNA Schwaetz and colleagues (2020), use carbon dots for the preparation of nano-formulation. They conduct experiment on *Nicotiana benthamiana* and *Solanum lycopersicum*. Through their experiments they sprayed nano formulation to obtain the GFP transgene silencing in both

plants. They also demonstrate the delivery efficacy of used nanoparticles by silencing of endogenous genes those are responsible for the silencing of the two subunits of a chlorophyll synthesis enzyme, magnesium chelatase. Their research provides evidence that the nano formulation can be used as efficient delivery tool in plant functional genomics.

METHODOLOGY

1. Identification of *MYMV* and its disease-causing gene:

1.1 Genomic DNA extraction

Virus symptoms containing leaves will be collected from field and used for the genomic DNA isolation via CTAB (Cetyl trimethyl ammonium bromide) method by Cota-Sánchez *et al.,* (2006) with require modifications.

1.2 PCR analysis of isolated DNA:

PCR analysis will be done using appropriate primer to confirm MYMV virus.

2. Construction of precursor siRNA: One step PCR approach will be used or the construction of siRNA as given by Voloudakis *et al., (*2015).

- The amplified target DNA will be used to mix with the components listed in the replicator RNAi kit and the procedure were followed which were present on the kit after obtain the resultant it will run on agarose gel electrophoresis.
- After the above procedure the obtained dsRNA will be purified via stepwise precipitation by following the procedure provided in the kit using lithium chloride solution.
- The pellet obtained in the above procedure will be washed using 70% ethanol for purification.

3. The biodegradable Nanoparticle formulation:

3.1 **PLGA nanoformulation**:

- Cationic poly(lactide-co-glycolide) nanoformulation will be prepared using the protocol given by Misra *et al., (*2014) using oil emulsion method with required modifications.
- The required concentration of PLGA polymer dissolves in emulsifying agent containing organic solution.

- The prepared emulsion will be allowed for sonication. The sonicated emulsion is placed on the magnet stirrer for the further process.
- The ultra-centrifugation which will be followed by lyophilisation facilitates the preparation of powdered NPs.

3.2 **Chitosan NPs preparation**

- Chitosan nanoparticles form by ionotropic gelation method by following the protocol given by calvo *et al.,* (1997) with required modification.
- Chitosan and TPP (Sodium tri polyphosphate) modified ration will be prepared and dissolved in acetic acid followed by placing on stirrer to obtain required pH.
- NP suspension will be used for morphological analysis.

3.3 **Characterisation of PLGA nanoparticle formulation:**

Different techniques used to characterise the physical and the chemical properties such as morphology, particle size, etc. using the techniques given by Misra *et al., (*2014) for PLGA and Calvo *et al.,* (1997) for chitosan with required modification.

4. Loading of dsRNA on PLGA Nanoparticles and Chitosan nanoparticles:

The siRNA will allow for surface adsorption using the method provided by Misra *et al., (*2014). The gel retardation assay will be used for PLGA and for loading of siRNA on Chitosan nanoparticles we will follow the appropriate protocol with required modification. The depolarised chitosan nanoparticles will be used to dissolve in sodium acetate buffer to obtain modified working solution range. Modified amount of siRNA will be added to filtered chitosan. SEM allows the characterisation of chitosan nanoparticles coated with siRNA.

5. Efficacy analysis of precursor siRNA loaded nanoparticles NPs against MYMV

For the quantification of mRNA level after delivery of siRNA loaded NPs, the total RNA extract from plant will be subjected to qRT-PCR (quantitative real time reverse transcriptase-polymerase chain reaction) (Mitter *et al.*,2003).

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